Relevant portions of NIH grant application

Figure 3 summarizes an experiment with SCC of the Lung, H-165, which was conducted to confirm that the Simplified Protocol utilizing only CldC and H_4U was as effective as the protocol utilizing 4 drugs. Average tumor volumes relative to day 0 are plotted vs days post initiation of treatment. The numbers in parenthesis indicate the number of tumors studied per group. The Standard Deviation was generally less than 10% of the mean. The total dose for four weeks of treatment was 52.5 Gy. After a bye of 3 weeks, treatment resumed on day 51 to day 60 with 2 courses of drugs and radiation. The additional dose of radiation in the ten day period was 22.5 Gy, resulting in a total dose of 75 Gy. The large arrow indicates the end of the initial 4 weeks of treatment. The smaller arrow indicates the initiation of the 10 day period of treatment. It is likely that better tumor control may have occurred with continuous rather than interrupted treatment. Treatment of H/N patients with tumors will involve 6 continuous weeks of RT.

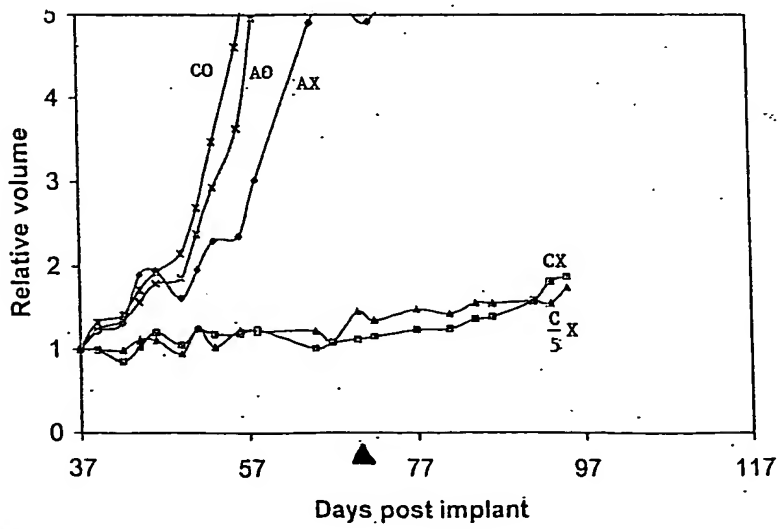
Note that there is no discernable effect of drugs on the growth rate of the tumors. The tumors of animals receiving radiation without drugs were in regrowth delay only during the weeks they were receiving radiation. The endpoints to reach 4× the initial tumor volume are 84, 112, 130, and 128 days for the irradiated groups AX, BX, CX and DX, respectively. Clearly the Simplified Protocols (Schedules C and D) are equal, if not superior, to the protocol utilizing 4 drugs, (Schedule B) as demonstrated with the prostate tumor, PC3. The transient moderate weight loss data for this experiment is shown in the publication (1).

The success of the protocols, utilizing only two drugs, may be attributed to the fact that CldUMP derived from CldC is a moderate inhibitor of thymidylate synthetase (Ki: FdUMP, CldUMP, BrdUMP, IdUMP = 0.014, 0.19, 1.4, and 1.6 μM, respectively (45), therefore, CldUMP derived from CldC not only overruns TTP, which competes with CldUTP for incorporation into DNA but interferes with the formation of TTP. Thus, the two inhibitors of the formation of TTP (PALA and FdC) are no longer necessary.

B. An approach to determine the minimal effective dose of CldC (Studies accomplished since the first application for STTR Phase 2 funding),

An experiment with PC-3 is summarized in Figure 4. There are 4 mice in the unirradiated groups and 7 mice in irradiated groups. A dose of 300 mg/kg of CldC/week is showing only slightly less tumor control than 1500 mg/kg/week (Schedule C) three weeks after the cessation of treatment with a total of 58 Gy over a five week period ending day 72 (arrow). Tumors treated with drug alone or with radiation alone average more than 5× their initial volume, respectively, whereas tumors treated with drug plus RT remained in tumor regrowth delay and average less than 1.6× their initial volume. There were 2/7 cures and 1/6 cures obtained with CldC at a dose of 60 and 300 mg/kg/day, respectively. This finding was confirmed in a second experiment utilizing a total dose of only 36 Gy over a 7 week period. The average tumor regrowth delay was identical for both groups with 1/6 cures occuring in each group. This is of importance because this dose of CldC (60 mg/kg/day) is the target endpoint dose in the Phase I trial: 2,230 mg/m^2/day. 60mg/kg/day ×37 (the conversion factor for mg/kg → mg/m^2) equals 2220 mg/m^2/day. (See Protocol, Appendix, item 4).

Figure 4.

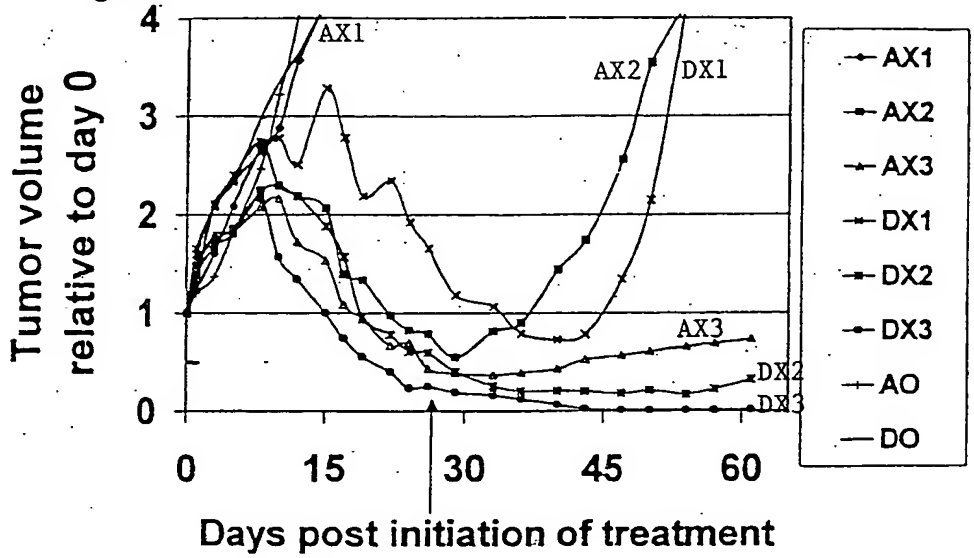


C. Studies examining the apparent CldC dose increase effect. Figure 5 summarizes an experiment in which we examined the radiation dose-increases effect of CldC+H_4U utilizing the human PC-3 tumor and Schedule D. The treatment was only four weeks. Total radiation administered over a 4 week period to different groups were: X1, 13 Gy; X2, 26 Gy; X3, 39 Gy. This is shown as Figure 7 in the publication (1).

Those tumors which were in tumor regrowth delay on day 60 were assigned a value of 60 at the last day of measurement. The days in tumor regrowth delay for AX1, AX2 and AX3 are 9, 40 and 51; the days in tumor regrowth delay for DX1, DX2 and DX3 are 47, 59 and 60, respectively. Note that AX1 is grouped together with the unirradiated control.

It appears that CldC+H_4U provides more than a two-fold dose increase effect in that DX-1 endpoints surpass those of AX-2. In the AX groups, mice developed secondary tumors at distant sites which accounted for 1 death in AX2 and one death in the AX3 group.

Figure 5. PC-3 Human Prostate Tumor



It is striking that a total dose of only 13 Gy resulted in a dramatic reduction in tumor volume in 5/7 tumors and a potential cure! (Compare AX1 to DX1 in figure 5).

The weight loss data shown in Figure 7a in the publication (1) in the Appendix (item 1) indicates that the moderate weight loss in drug treated groups receiving radiation was substantially recovered each weekend and fully recovered once treatment stopped. The results was essentially similar to that shown for PC3 in Figure 2'.

Figure 6. HTB-43 Human Hypopharyngeal SCC

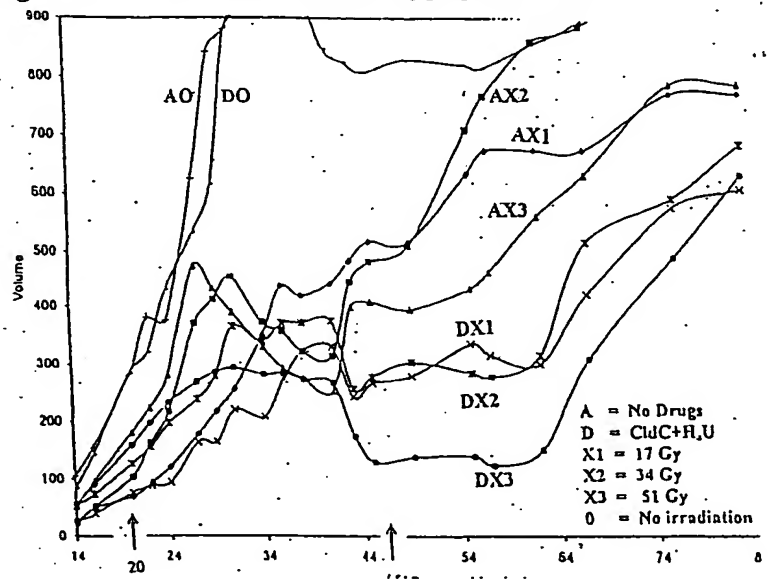


Figure 7. HTB-43 Human Hypopharyngeal SCC

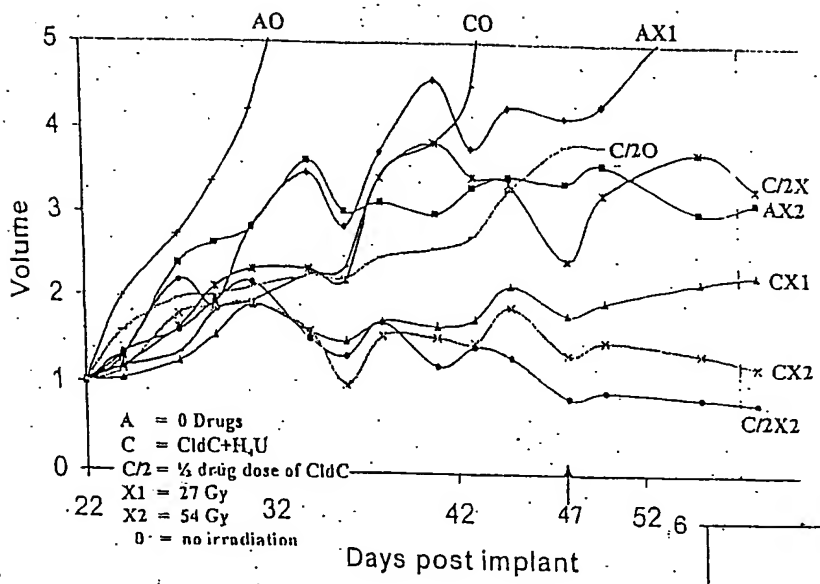
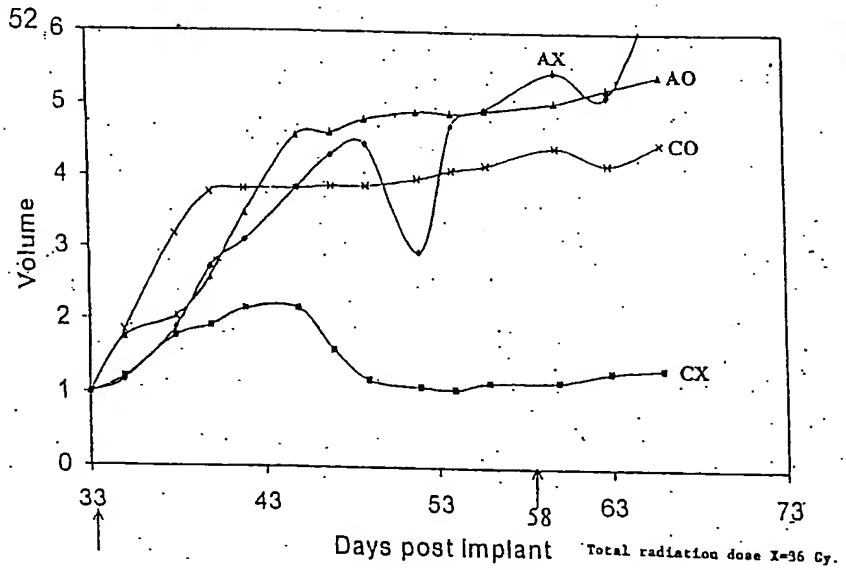


Figure 8. SCC-6 Human SCC of the Tongue



D. Recent tumor inhibition studies with human H/N tumors. (Studies accomplished since the first application for STTR Phase 2 funding). Figures 6-8 summarize recent initial studies with two human H/N tumors. Unirradiated groups contained 4 mice; irradiated groups contained 7 mice. Treatments were for 4 weeks. The tumor inhibition studies are correlated below with a) enzymatic studies, b) % of cells of human tumors and normal mouse tissue incorporating ClUra in their DNA and c) the % replacement of thymine by ClUra (Table 2).

Figure 6, a study utilizing HTB-43, a human hypopharyngeal SCC, utilized schedule D and four weeks of treatment starting on day 20 post implantation. The average tumor volumes are shown. Although the tumor control was transient and no cures were obtained, it can be seen that once treatment was terminated, the tumors receiving radiation only groups (AX) began accelerated growth. Tumor regrowth delay continued for more than two weeks in all DX groups (which received drugs and radiation). Of considerable importance is the fact that DX-1 was superior not only to AX-1 and AX-2, but it was superior to AX-3, indicating the potential of an apparent 3-fold dose increase effect which we encountered in cell culture and with the mammary adenocarcinoma EMT-6 in our past studies (30).

Figure 7 summarizes a second experiment with HTB-43 in which CldC was administered according to Schedule C. We also examined the effect of CldC at a dose of 750 mg/kg/week (C/2). The relative tumor volumes are shown. The inhibition of tumor growth by C/2 O, which was greater than CO was atypical. This

tumor grows like a hard bead and occasionally breaks open. Dr. David Arnold, a surgeon, commented that this is often a characteristic of H/N patient tumors. In spite of the problems with this tumor, we were able to demonstrate that CX-1 was substantially more effective than AX-2 (and far more effective than AX-1). Although a two-fold dilution of drug was utilized (C/2), at a total dose of 54 Gy, the tumor inhibition was as effective as CX2 (½ X2 = CX2) and much more effective than CX1 (27 total Gy of radiation). It is not surprising that the lower dose of CldC (½) requires a higher dose of radiation to manifest substantial tumor control above that displayed without drug. Because so many drugs fail because they are only effective at high doses, the studies in these nude mouse models, utilizing lower doses of drug, are most relevant to future clinical practice – even though CldC appears not to be toxic at high doses.

Figure 8 is a preliminary study which shows that SCC-6, a tumor of the tongue, is radiosensitized to a great extent by CldC at a dose of 1500 mg/kg of CldC/week for four weeks with a total dose of 36 Gy. Relative average tumor volumes are plotted. The Standard Error for experiments summarized in Figs. 6-8 was less than 15%.

E. Enzymological Studies

1. Studies with patients with H/N tumors. Table 1 summarizes the results of enzymatic studies with the normal and tumor tissue of 24 patients with head and neck tumors (including thyroid). A table with more details appears in the publication (1) (as Table 3) in the Appendix. The patient numbers correspond to the numbers in the publication. Both enzymatic assays could be conducted with as little as 25-30 mg of tissue. In 14/24 tumors, dCK is elevated greater than 3-fold (3 to 17×), in 11/24 tumors both enzymes are elevated greater than 3-fold over normal tissue. The elevation of dCMPD is most critical for the strategy. It is elevated more than 6-fold in 10/24 tumors. Starting with tumor 14, the study was conducted as a blind study because of the remarkable nature of the results. In the paper by Guisti et al (4) on which this strategy is based, two malignant head and neck tumors were also found to have high levels of dCMP deaminase: carcinoma of the buccal mucosa and adenocarcinoma of the floor the mouth. These levels were approximately 7× higher than the levels in 3 benign tumors: fibroma of the gum and lips, papilloma of the palantine mucosa and chrondroma of the jaw.

An analysis of the data in Table 3 of the publication shows that the range of actual values of enzyme activities are not widely scattered. With an arbitrary cut-off of 25 units of dCK activity, only 2/25 of the normal tissue are over the cut-off, whereas 18/26 tumor tissues are over the cut-off. With respect to dCMPD activity, using a cut-off value of 10 units, only 2/25 of the normal tissues are above the cut-off and 21/26 of the tumor tissues are above the cut-off. Therefore, not only are the T/N ratios impressive, but the distribution of the actual enzyme activities are encouraging for future clinical studies.

2. Studies of patients with tumors of the lung and rectum (Studies accomplished since the first application for STTR Phase 2 funding). Elevation of both dCK and dCMPD in patients with SCC of the lung over that of normal lung tissue does not appear to be as frequent as those encountered with H/N tumors (8/35 compared to 12/24). Several of the 8 patients had substantially elevated levels of the critical enzymes in their tumor over that of normal adjacent tissue. For example: patient X T/N: dCK = 7, dCMPD = 17.

A preliminary study of only 4 rectal tumors shows that two of them have elevated levels of dCK and dCMPD over that of adjacent normal tissue: patient A, T/N: dCK = 3, dCMPD = 36 and patient B, T/N: dCK = 12, dCMPD = 29.

Table 1 A Summary of Relative Enzymatic Activities

Patient	Tumor Site	dCK T/N	dCMPD T/N	Patient	Tumor Site	dCK T/N	dCMPD T/N
1	Larynx	1.4	30	15	Oral Cavity	2.6	2.5
3	"	2.3	5.8	16	Tongue	11	4.4
5	"	2.8	9.5	17	"	5.1	6.1
6	"	1.2	2.9	18	Oropharynx	9.4	26
7	"	0.71	4.8	19	Maxilla	1.9	3.7
8	"	3.1	3.1	20	Parotid	16	8.4
9	Oral Cavity	3.1	16	21	Submandibular	9.5	20
10	"	4.6	9.7	22	Facial Skin	4.1	2.2
11	"	17	20	24	Hypopharynx	5.5	2.7
12	"	2.4	1.9	25	Thyroid	1.1	2.4
13	"	1.4	12	26	"	4.8	140
14	"	7.1	2.2	27	"	5.6	1.6

3. Enzymatic studies with human tumor cell or tissue lines being utilized in the nude mouse studies. The levels of dCK in the PC3 human prostate tumor and H-165, the human squamous cell carcinoma of the lung are 16.2 and 35.5 units, respectively. The levels of dCMP deaminase in the PC3 and H-165 human tumors are 3.63 and 14.1 units, respectively. With PC3, the levels of both dCK and dCMPD are more than 3× higher than ⅓ of the normal tissues of the patients with H/N tumors in the publication (1) in the Appendix. The levels of dCK and dCMPD in the H-165 tumor are more than 3× higher than ½, and ¾, respectively, of the normal tissues of the patients. These latter results with the human prostate and lung tumors will have greater meaning when comparisons are made with normal urogenital and rectal tissue as well as normal lung tissue.

We have determined the levels of dCK and dCMPD in 3 H/N tumors (since the first application for STTR Phase 2 funding). The enzyme assays were performed on the solid tumor HTB-43, a hypopharyngeal SCC obtained from the NIH as tissue fragments. SCC-1 and SCC-6 were assayed from cell culture where the low levels of protein may contribute, in part, to the high apparent activities. SCC-1 a tumor of the floor of the mouth, has not been studied further at this point; SCC-6 is a tumor of the tongue. HTB-43 and SCC-6 were utilized in preliminary tumor inhibition studies (Figures 6-8) and were the objects of studies utilizing FACS and HPLC analysis (Table 2). Because we are comparing activities in human tumors to mouse tissues, this may be an 'apples to oranges' situation except for the possibility that it is important to note a general correlation between enzyme activity, labeling index and the presence or absence of pathology of the tissue.

F. The % of tumor cells incorporating ClUra in DNA (% LI). In a collaborative study with Drs. Allan Pollack and Nicholas Terry, Professors of Radiation Oncology at the MD Anderson Cancer Center, unirradiated mice bearing human PC3 prostate tumors were treated with Cytochlor + H_4U with Schedule D (escalating doses of CldC 10% each week). The % of tumor cells containing ClUra in DNA by FACS analysis was: 51%, 78%, on days 3 and 5 of week one and 92% and 92% on days 3 and 5 of week two, respectively. Dr. Nicholas Terry has recently (since the first application for STTR Phase 2 funding) provided the data indicating the % of cells of two H/N tumors which have incorporated ClUra derived from CldC in their DNA. These results are shown in Table 2. Of interest is the fact that 99.5% labeling of HTB-43 occurred with ½ of the dose of CldC (750 mg/kg/week) after two weeks of treatment. The average % of LI for the tongue of treated nude mice was 59%